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GRANT NUMBER DAMD17-94-J-4040

TITLE: Mapping Mammary Carcinoma Suppressor Genes in the
Laboratory Rat

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REPORT DATE: July 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 2

19980130 175

REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Final (1 Jul 94 - 30 Jun 97)	
4. TITLE AND SUBTITLE Mapping Mammary Carcinoma Suppressor Genes in the Laboratory Rat			5. FUNDING NUMBERS DAMD17-94-J-4040	
6. AUTHOR(S) Michael Gould, Ph.D. Hong Lan, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin Madison, Wisconsin 53706			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, MD 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Inbred strains of rats differ in their susceptibility to both spontaneous and chemically-induced mammary carcinogenesis. We have been using genetic breeding strategies between resistant and susceptible rat strains, followed by linkage analysis to identify the mammary carcinoma susceptibility (<i>Mcs</i>) gene(s) responsible for the mammary carcinoma resistance trait in the inbred Copenhagen (Cop) rat. Simple sequence repeat (SSR) markers from the literature as well as from our chromosome-specific libraries were used to map the Cop rat genome and fine-map the regions involved in the resistance phenotype. So far we have found 4 loci (3 resistance and 1 enhancer) that show linkage with the mammary carcinoma resistance phenotype. The locus on chromosome 2 (termed <i>Mcs1</i>) is believed to contain the major tumor resistance gene, while other loci such as <i>Mcs2</i> on chromosome 7, <i>Mcs3</i> on chromosome 1, and <i>Mcs4</i> on chromosome 8, represent relatively weaker genes.				
14. SUBJECT TERMS Breast Cancer Susceptibility, Mammary Epithelium, Gene Mapping, Loss of Heterozygosity, Tumor Suppressor Genes, Mammary Specific			15. NUMBER OF PAGES 57	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION

It has been known since the 1950's that different strains of laboratory rats exhibit different susceptibility to various kinds of tumors (Dunning and Curtis, 1945). The Wistar Furth (WF) strain is highly susceptible to both spontaneous and chemically-induced mammary carcinomas. The Fischer 344 (F344) strain exhibits intermediate susceptibility. The Copenhagen (Cop) and Wistar Kyoto (WKy) strains are resistant to both types of mammary cancer (Dunning and Curtis, 1945; Gould, 1986; Haag *et al.*, 1992). The resistance phenotype of the Cop rat to mammary carcinogenesis was initially described in the 1940's. Using the chemical carcinogen 2-acetylaminoflourene (AAF), it was shown that Cop rats rarely developed mammary cancers but were not protected from the formation of hepatic cancers. Thus, the cancer resistance phenotype of the Cop rat was believed to be mammary specific (Dunning and Curtis, 1945). Research completed within the last ten years by both this laboratory and that of Dr. John Isaacs pinpointed the nature and site of action of the genes involved in this phenotype. Using classical genetic breeding studies and transplantation studies, it was demonstrated that the mammary carcinoma resistance phenotype of the Cop rat was likely due to a single autosomal dominant gene whose site of action lies within the mammary epithelium (Isaacs, 1986; Gould *et al.*, 1989; Zhang *et al.*, 1990).

The goal of this research is to map and eventually clone the rat gene(s) responsible for the tumor resistance phenotype. The gene is termed *Mcs* (mammary carcinoma susceptibility, which can be high or low depending on whether the gene

confers a resistance or enhancer phenotype). When it refers to a resistance gene, it can also be called mammary carcinoma suppressor gene, the term we used in our original proposal. We hope the gene(s) will prove useful as diagnostic indicators of breast cancer risk in humans and possibly lead to the development of new drugs for the treatment of human breast cancers. Initial work had already been completed when this project was proposed. Using four strains of rats; two of which are susceptible to chemically-induced mammary carcinogenesis (WF and F344) and two of which are resistant (Cop and WKy), two separate backcross sets of animals were generated and tested for the development of mammary tumors following administration of 7,12-dimethylbenz (a) anthracene (DMBA). A genome-wide scan of the *Mcs* phenotype-related locus using simple sequence repeat (SSR) was performed in the (WF X Cop)F1 X WF backcross (183 animals) using tumor number as a quantitative trait, in order to allow for the possibility that resistance could be due to more than one gene locus (Lander *et al.*, 1987). About 50%-70% of the genome was covered in the initial scan using a total of 114 polymorphic markers. The minisatellite marker M13 (D2Uwm1) was shown to be linked to the *Mcs* phenotype. This locus (termed *Mcs1*) was tentatively located within a 40 cM interval at the centromeric end of chromosome 2. Fluorescence *in situ* hybridization (FISH) to whole rat chromosomes using a P1 clone containing the sequence of marker D2Mit1 (R1025), which maps near D2Uwm1, confirmed mapping to 2q1 (Hsu *et al.*, 1994). D2Uwm1 yielded a lod score of 3.8. It is now suggested that for a genome-wide scan of mouse or rat loci underlying complex traits in a backcross mapping

panel a lod score threshold of 1.9 should be reported as "suggestive linkage" while that over 3.3 should be treated as "significant linkage" (Lander and Kruglyak, 1995). To confirm a linkage in an independent cross, a pointwise p value of 0.01 is required (Lander and Kruglyak, 1995).

The original objectives of the proposed research were as follows:

1. Isolate new simple sequence repeat markers to fine-map (to within 1 cM resolution) the region of chromosome 2 surrounding the *Mcs1* locus.
2. Test additional markers over the thus far untested regions of the rat genome for linkage to the *Mcs* phenotype (to 5 cM resolution). Confirm mapping of additional genes to specific chromosomes by FISH to whole chromosomes using specific SSR-positive P1 clones.
3. Fine-map new areas in the genome (to 1 cM resolution) that demonstrate linkage to the *Mcs* phenotype. Begin to positionally clone the gene(s) identified.
4. Isolate coding sequences from P1 or cosmid clones containing *Mcs* phenotype-linked SSRs for determination of homologous regions in the human genome and possible correlation with already identified human genes.
5. Test for loss of heterozygosity (LOH) of *Mcs*-linked SSR markers in mammary tumors of two independent F1 hybrids following radiation and DMBA tumor induction.

In the past three years, we have identified three additional loci, *Mcs2* on chromosome 7, *Mcs3* on chromosome 1, and *Mcs4* on chromosome 8 from the genome-wide scan of the (WF X Cop)F1 X WF backcross. These loci were fine-

mapped with SSR markers isolated from chromosome-specific libraries as proposed for *Mcs1*. The new SSR markers isolated from our chromosome-specific libraries were initially called UW (University of Wisconsin) markers.

There have been changes in the name of rat genetics markers (<http://ratmap.gen.gu.se/ratmap/WWWNomen/Brief.html>). Rat SSR markers were initially recorded as Rxxx (xxx are numbers) originally, but they are now inventoried as DxOrgx (the first x is chromosome number; Org is the code of institution where the marker is generated, for example, UW markers generated in this laboratory are DxUwmx; the last x is number). In this report, we keep the R-symbol system for markers in the appendix because this project started a long time ago and early results were reported by R-symbol. But we use the D-symbol system for markers mentioned in the text.

It should be noted at this point that all of the data reported in this document is a result of the work of a group of investigators. Dr. Hong Lan (successor to Dr. Virginia Ford, the original fellowship recipient) has focused his efforts over the last two years on the fine-mapping of *Mcs2* and *Mcs3*. While Dr. Lan did not specifically conduct the work on *Mcs1* and the LOH studies, he was involved on a weekly basis with the progress and design of these experiments. As with most large mapping projects, this one was a team effort.

BODY

MATERIALS AND METHODS

Generation of Chromosome-Specific Libraries

We have worked with several protocols for generating chromosome-specific libraries and here we present the one we think works best. The modifications we made will be discussed in more detail in the Results and Discussion section.

For chromosome-specific-libraries, the DNA sources were obtained by flow-cytometric sorting of rat chromosomes (Shepel *et al*, 1994), and for each reaction, about 300-400 sorted chromosomes were used directly for DOP-PCR without DNA purification.

DOP-PCR primers were either:

6-MW: 5'CCGACTCGAGNNNNNNATGTGG3' (Telenius *et al*, 1992), or two primers that we modified:

ATCTGC: CCG ACT CGA GNN NNN NAT CTG C

ATCAGC: CCG ACT CGA GNN NNN NAT CAG C.

The template for each reaction consisted of 300-400 copies of whole sorted chromosomes and 100 pg (~30 genome equivalents) of Copenhagen DNA as a positive control. A negative control reaction containing no DNA was also included to insure no background contamination. Final concentrations of reagents were 10 mM Tris, pH 8.3, 50 mM KCl, 3.0 mM MgCl₂, 0.001% gelatin, 200 mM dNTPs, 1.5 mM primer, 1.25 Units Taq LD polymerase (Perkin-Elmer Cetus) in a final volume of 50

μl. Note also that for sorted chromosomes, there will be additional components in the reaction which come from the sheath fluid in which the chromosomes are sorted (some salts and spermine and spermidine), but these did not seem to inhibit the reaction. The reaction mixtures were first heated to 95°C for 5 min, followed by 10 cycles at 94°C for 1 min, 30°C for 1.5 min, 30°C-72°C for 3 min, and 72°C for 3 min, then followed by 20 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min with 1 second added to the 72°C step per cycle. A further 72°C synthesis step was carried out for 10 min after the cycles were done. After DOP-PCR, 5 μl aliquot from each reaction was subject to 1.5% agarose gel electrophoresis to check the PCR product under ultraviolet light following ethidium-bromide staining. If both the controls and the reactions worked well, half of each PCR reaction was then used for hybridization-selection/affinity capture as described below.

In order to isolate SSR markers more effectively, some modifications have also been made to the method we presented in the initial proposal for cloning the DOP-PCR products. Before cloning, the DOP-PCR products were enriched for (CA)_n repeats by hybridization-selection followed by affinity capture on Affinitips™ (a pipette tip micro-column for streptavidin capture of biotinylated molecules; Genosys). In this procedure, excessive biotin-labeled (CA)_n probe (200 pmol) was hybridized with 20 μl denatured DOP-PCR products in 50 μl phosphate-SDS solution (0.5 M NaPO₄, pH7.4, 0.5 % SDS) in an eppendorf tube at 50°C for 2 hrs. The PCR products that contain (CA)_n repeats anneal to the biotinylated probe. Then the hybridization mix was applied to the Affinitip column and the PCR products were

bound and washed according to manufacturer's specifications. The single-stranded target fragments containing (CA)_n repeats were eluted with ddH₂O at 65°C, then precipitated with ethanol and resuspended in 5 µl ddH₂O. This step enriched the library for (CA)_n-containing clones by about 150-fold. We found that 10-80% clones in the final library were positive for (CA)_n repeats after this hybridization selection.

To prepare the (CA)_n-enriched products for insertion into the pAMP10 vector (Life Technologies), the eluted DNA was re-amplified with the DOP-PCR primer bearing a (CUA)₄ tail at the 5' end. The 20 µl reaction was heated to 94°C for 5 min followed by 15 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The final synthesis step was extended to 10 min. Two µl of this product were annealed into the pAMP10 vector (20 µl reaction volume) according to the manufacturer's directions (CloneAmp Systems, Life Technologies). One µl of the annealed reaction was used to transform DH5αF' library efficiency competent cells (Life Technologies) yielding 50-4000 transformants grown under ampicillin selection.

Screening and Sequencing of Chromosome-Specific Libraries.

Transformants were further screened for (CA)_n repeats by a simple PCR-based method we call Colony PCR. One µl from a bacterial culture was used directly as DNA template. Each clone was subjected to two 10 µl PCR reactions: one with M13 forward and reverse primers to estimate the insert size, the other with M13 forward and reverse primers plus an additional N(CA)₁₀ primer to determine whether there was (CA)_n repeat and how far the repeat was away from the M13 primer. In the M13

forward + reverse + N(CA)₁₀ PCR, the N(CA)₁₀ primer annealed to (CA)_n repeats in the insert sequence and hence gave a PCR product with either the M13 forward or reverse primer. The random base at the 5' end of the N(CA)₁₀ primer served to anchor the primer at the end of the repeat sequence in order to avoid multiple priming along the repeats. Each reaction contained 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM dNTPs, 2.0 μM each primer, 0.25 Units Taq polymerase (Perkin-Elmer Cetus). The mixture was heated to 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 30 sec. The final synthesis step was extended to 7 min. For screening multiple clones, the PCR was set up in 96-well plates using a multichannel Rainin motorized pipette, overlaid with light mineral oil (Sigma) and run on an MJ Research PTC-100 Programmable Thermal Cycler. The PCR products were run on a 2% agarose gel and clones that showed an extra band in the M13 forward + reverse + N(CA)₁₀ PCR were considered positive clones that contained (CA)_n repeats.

Positive clones with suitable insert sizes were isolated and plasmid DNA for sequencing was extracted by alkaline-lysis method using the QIAGEN plasmid purification system (Qiagen, Inc). DNA sequencing to identify the unique DNA sequences surrounding the (CA)_n repeats was performed using the PRISM Dye-Terminator fluorescent sequencing system (Applied Biosystems Incorporated-Perkin Elmer), and was performed at the University of Wisconsin Biotechnology Center. The insert sequence data were analyzed either by the computer program GCG Version 8.2 (University of Wisconsin-Madison), or by DNASTar (DNASTar, Inc) and

Oligo 5.0 (National Biosciences Inc.). New primers were generated spanning the repeats and were analyzed in the four rat strains to determine which markers were informative (ie., polymorphic) in our parental strains.

To measure the length variation of the SSR, genomic DNA was used as a template for PCR and PCR products were run on an 2.5-3.5% MetaPhor® agarose (FMC BioProducts) gel. The gel was stained with SYBR green I (FMC BioProducts or Molecular Probes) and visualized using a FluoroImager (Molecular Dynamics). For markers in which strain variations were too small to be detected by agarose gel, radiolabeled deoxynucleotide was incorporated in the PCR reaction so that the product could be visualized by autoradiography or phosphorimaging (Molecular Dynamics) after electrophoresis on a polyacrylamide sequencing gel. Informative markers were then genotyped in the backcross animals for linkage to the *Mcs* phenotype.

Linkage Analysis of SSR Markers

Genomic DNA samples were prepared from either tails or spleens of the backcross and parental animals using the standard procedure (Ausubel *et al.*, 1987). SSR marker primers were synthesized by (for markers we generated) or ordered from (for markers commercially available) Research Genetics. Genotyping was performed in 5 µl reactions containing 50 ng genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 120 pmol of each primer, and AmpliTaq polymerase (0.5-1 U/100 microliters, Perkin Elmer Cetus). The reactions

were set up in 96-well plates (MJ Research Inc.) using a motorized microliter pipette (Rainin Instrument Co. Inc.), overlaid with light mineral oil (Sigma) and run on Programmable Thermal Cycler PTC-100 (MJ Research Inc.) using the following cycling conditions: denaturation at 94°C for 3 min followed by 30 cycles of 95°C, 1 min, 55°C, 1 min, and 72°C, 30 sec. A final 72°C extension step was carried out for 5 min. PCR products were run on an 2.5-3.5% MetaPhor[®] agarose (FMC BioProducts) gel in 1 X TBE buffer (89 mM Tris-Borate, 2.5 mM EDTA, pH8.3), on an HE 99X Max Submarine Electrophoresis Unit (Pharmacia Biotech). The gel concentration depended on the size of the PCR products. After electrophoresis, the gel was stained with 1:30,000 diluted SYBR green I (FMC BioProducts or Molecular Probes) in H₂O in a plastic container for 10 -40 min and then visualized using a FluorImager (Molecular Dynamics). The signal was analyzed by the computer software ImageQuant (Molecular Dynamics). For markers in which strain variations were too small to be detected by agarose gel, PCR reactions were performed with [α -³²P]dATP (3000 Ci/mmol) and resolved on 5% polyacrylamide sequencing gels. For each reaction 50 ng of genomic DNA was amplified in a 5 μ l reaction containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 120 nM of each primer, 200 μ M dNTPs, 0.14 μ Ci of [α -³²P]dATP, and 0.25 U AmpliTaq polymerase. The reactions were set up in 96-well plates using a Biomek workstation (Beckman Instruments), overlaid with mineral oil and run on a Programable Thermal Cycler PTC-100 using the following cycling conditions: denaturation at 94°C for 3 min followed by 25 cycles of 95°C, 1 min; 55°C, 1 min; and 72°C, 30 sec. A final 72°C

extension step was carried out for 5 min. Wet gels were transferred to Whatman 3MM paper, wrapped in plastic wrap, exposed to either film or a phosphorimager screen (Molecular Dynamics) followed by analysis by eye or the ImageQuant software respectively.

Genotypes and phenotypes of backcross animals were subjected to linkage analysis using the MAPMAKER computer program (Lander *et al.*, 1987) and a quantitative analysis was performed with the program MAPMAKER-QTL (Lander and Botstein, 1989). In the quantitative analysis, the square root transformation (Dietrich *et al.*, 1993) of the tumor number is dealt with as a quantitative trait and estimates the contribution of a given locus to the phenotype. Alternatively, Qlink (Drinkwater, University of Wisconsin-Madison), a newly written program to simplify linkage analysis for quantitative trait loci using the nonparametric methods based on those described by Kruglyak and Lander (1995) was also used to estimate the lod score and *p* value at a given marker position. All the lod scores in this report were calculated by Qlink unless otherwise specified.

For the genome-wide scan in the (WF X Cop)F1 X WF backcross (183 animals), 90 rats having the highest and lowest number of carcinomas were chosen for initial genotype analysis in order to reduce the number of progeny to be genotyped. Selecting these extremes increase the odds of locating genetic linkage to the phenotype (Lander and Botstein, 1989). Any regions having a lod score of 1.0 or greater with this panel were then genotyped using the remaining 93 DNA samples from animals having an intermediate number of tumors.

Test for Loss of Heterozygosity of *Mcs*-Linked SSR Markers

In order to identify and compare the genetic lesions associated with tumorigenesis in rats carrying *Mcs* genes, we induced mammary carcinomas in (WF X Cop)F1 rats using either DMBA or radiation. The tumors were screened for allelic imbalance using PCR and polymorphic SSR markers spanning the genome.

DNA was isolated from tumors by proteinase K digestion and phenol: chloroform extraction. For each DNA sample, the total PCR volume was 5 μ l, consisting of 2.5 μ l of 20 ng/ μ l DNA and 2.5 μ l of PCR master mix. Final concentrations in the PCR reaction mixture were 1 X PCR buffer, 200 μ M dCTP, dTTP, dGTP, 20 μ M dATP, 0.12 μ M each primer, 0.25 U of AmpliTaq DNA polymerase (Perkin-Elmer) and 0.025 μ l [α -³²P]ATP (DuPont NEN, specific activity 3000 Ci/mmol, 10 mCi/ml). A Biomek 1000 or 2000 automated laboratory workstation (Beckman) was used to set up the PCR reactions in 96-well plates. The PCR cycling consisted of 94°C for 3 min followed by 25 cycles of 94°C for 1 min; 55°C for 1 min; and 72°C for 30 sec. A final 72°C extension step was carried out for 5 min. The samples were heat-denatured, mixed with 4 μ l loading dye, loaded on 5% sequencing gels and resolved. Gels were exposed to a phosphor screen, scanned using a PhosphorImager (Molecular Dynamics), and analyzed quantitatively using ImageQuant software. An allelic imbalance, such as loss of heterozygosity or gene duplication was defined as a 25% or greater difference in the amount of radiolabel incorporation into the PCR products for the individual alleles after normalization

to an F1 control spleen DNA sample. SSR instability was identified as any change in allele sizes. All allelic imbalances initially observed were confirmed by repeating the PCR and quantitative analysis at least one time.

RESULTS AND DISCUSSION

Objective 1. Isolate new simple sequence repeat markers to fine-map (to within 1 cM resolution) the region of chromosome 2 surrounding the *Mcs1* locus.

The centromeric end of rat chromosome 2 is one of several poorly mapped areas in the rat genome so that markers are not readily available for fine-mapping the *Mcs1* locus. The *Mcs1* locus was defined in a 40 cM lod-1 region with the only marker available when this project started (D2Uwm1). We have tried in the past three years to isolate new SSR markers from chromosome-2-specific libraries to fine-map the *Mcs1* region, and have recently successfully developed the methodology. In addition, microsatellite markers from various commercial and collaborative sources were used. We have 10 new markers around the *Mcs1* region; four of which (D2Mit29, D2Rat2, D2Rat3, and D2Wox2) were from the literature, one (IP13DIS) was generated from a newly published rat gene sequence, and 5 were markers we generated from our chromosome-2 specific libraries. Using this relatively dense genetic map, MAPMAKER/QTL analysis with square-root of the tumor number as the phenotype yielded a peak lod score of 4.1 near marker D2Uwm14 (≈ 1 cM from

D2Uwm1) with a lod-1 support interval of approximately 8 cM from the centromere of the chromosome to marker D2Uwm16 (Figure 1).

As we mentioned in the 1995 annual report, we failed to generate a chromosome-2-specific library from micro-dissected chromosome fragments or by using the GDRDA (Genetically Driven Representational Difference Analysis) technique. The new markers were generated from libraries constructed from flow-sorted chromosomes. The rat chromosome 2 is large, so the new SSR markers reported here represent only a small number of UW markers from the chromosome-2 specific libraries. When we obtained the 5 UW markers in our target region, we had 14 markers mapped to other parts of the chromosome and 11 mapped to other chromosomes.

It should be pointed out that we found a gene marker (IP13DIS, NADH ubiquinone oxidoreductase subunit (IP13) gene, GenBank access no. L38439) which maps about 6 cM away from *Mcs1*. This is the only gene that was identified close to the *Mcs1* region on rat chromosome 2. This marker should be very useful when looking for the homologous regions of *Mcs1* in the human and mouse genomes, although the gene itself is not mapped in either the human or the mouse.

In order to better define *Mcs1* and to confirm its mapping position, two additional independent rat crosses were generated and tested for linkage as was done for the (WF X Cop)F1 X WF backcross (183 animals) (BC1). We first generated a (WF X Cop)F1 X (WF X Cop)F1 intercross mapping panel (250 animals) (F2) and induced tumors in F2 animals with DMBA. Then we generated a second backcross (BC2)

which contained 417 female animals that were treated with DMBA. DNA samples from all of the animals in F2 and BC2 were genotyped for markers in the lod-1 intervals of the *Mcs1* and other *Mcs* loci. A combined analysis of data from all the three crosses was performed to better define the peak markers using the method in the QLink program developed by Drinkwater (1997). This analysis yielded a peak lod score of 15.2 near D2Uwm14, indicating that there is clearly a mammary carcinoma resistance locus, *Mcs1*, near the centromeric end of rat chromosome 2. We are about to start physical mapping of this locus so that we may positionally clone the *Mcs1* gene in the future.

Objective 2. Test additional markers over the thus far untested regions of the rat genome for linkage to the *Mcs* phenotype (to 5 cM resolution). Confirm mapping of additional genes to specific chromosomes by FISH to whole chromosomes using specific SSR-positive P1 clones.

During the past three years, there has been great progress in the Rat Genome Project at the Whitehead Institute/MIT Center for Genome Research. The number of SSR markers available for rat gene mapping had increased to about 2000 by June, 1997 (<http://www.genome.wi.mit.edu/rat>). Those that are informative have been genotyped in BC1 and were added to our overall map of the genome and have been tested for linkage to the *Mcs* phenotype. The updated genome map shown in the Appendix contains 340 markers compared with the 114-marker map at the time the project started. If the size of the rat genome is estimated as 2250 cM

(<http://ratmap.gen.gu.se/chromap.html>), the average distance between two markers on our map is about 6.6 cM.

The genome-wide scan in BC1 initially revealed three additional loci with lod scores ≥ 1.0 on chromosomes 7, 1, and 8, respectively. We then added markers to each chromosomal map to more densely cover those regions, and tested the full panel of 183 animals using all markers. As the density of the rat genetic map has improved in recent years, we did not confirm mapping of these loci to specific chromosomes by FISH to whole chromosomes using specific SSR-positive P1 clones. Instead, we tested the linkage of these loci in other independently generated mapping panels and performed combined analysis as was done for *Mcs1* to confirm the existence of these loci. We found three other loci, *Mcs2* on chromosome 7, *Mcs3* on chromosome 1 and *Mcs4* on chromosome 8 that show linkage to the resistance phenotype (see Objective 3 for more details).

Objective 3. Fine-map new areas in the genome (to 1 cM resolution) that demonstrate linkage to the *Mcs* phenotype. Begin to positionally clone the gene(s) identified.

Subtopic 1. *Mcs2*

We made several chromosome-7-specific libraries and isolated new SSR markers (we called UW markers) from these libraries. There are 17 polymorphic

markers added to the chromosome 7 map of the (WF X Cop)F1 X WF backcross. The marker that shows the highest lod score is D7Uwm8 (lod score 3.5) (Figure 1).

However, in the F2 mapping panel (250 animals), no linkage was found between *Mcs2* markers and the tumor resistance phenotype. As a significant lod score of 3.3 indicates that the probability of a false positive event (due to statistics fluctuation) in a genome-wide scan is less than 5%, it is unclear why this locus, with a lod score of 3.5 in the backcross, did not show any linkage in the intercross. We then tested the markers in BC2 (with 417 animals) and the linkage showed up again. A combined analysis of data from all the three crosses showed that *Mcs2* has a peak combined lod of 7.9 but was within a large interval of more than 36 cM.

While we constructed chromosome-7-specific libraries, we made some modifications to our original protocol to improve the effectiveness of generating new SSR markers. As we mentioned in the annual report in 1996, there had been some problems with the first chromosome-7-specific library we analyzed in 1995-1996:

- 1) It seemed that unique clones from the library were limited. At the initial screening stage, most of the clones in the library were unique. However, as more clones were sequenced, fewer clones appeared to be unique. After 200 to 300 clones had been sequenced, we could only get one unique sequence from every 2 or 3 clones, thus we assumed that the library was short of novel unique clones. As there was not a simple method to tell whether a clone was unique prior to sequencing,

and sequencing more and more duplicate clones was costly, we moved on to new libraries.

2) Many clones from the library contained inserts that had repeats at or near the end of the insert, therefore we were usually not able to pick up enough flanking sequences for PCR primer design. We assumed this may have resulted from the DOP-PCR primer used in making the library. The 3' end of the primer, TGTGG, might easily anneal to (CA)_n repeat regions during the low annealing temperature DOP-PCR stage and thus might amplify many sequences with (CA)_n repeats at one end.

3) Contamination in the DOP-PCR was usually a problem too. In the protocol we reported last year, two rounds of PCR were needed to accumulate enough products for hybridization selection of SSR fragment, as observed by another group (Gu *et al.*, 1996). As there are 10 cycles with an annealing temperature of about 30°C in the DOP-PCR protocol, any tiny contamination of the DNA source will be amplified. Therefore, after two rounds of PCR, the no-DNA control from the first round of PCR was usually contaminated. The technical difficulty in performing DOP-PCR usually limits its application to genetic studies.

When we made new libraries in the past year, we deliberately took efforts to improve the DOP-PCR. First, we modified the 3' end of the commercially used 6-MW DOP-PCR primer. We designed several 3' ends which would not bind to (CA)_n repeats during PCR, and checked their abundance in the rat genome by searching for

their occurrence among the rat DNA sequences that had been submitted to GenBank by June of 1996. We found the following result:

3' end	Occurrence
ATGTCC	4025
ATGTCG	1026
ATGTGC	3360
ATGTCG	1336
<u>ATCTGC</u>	<u>4397</u>
ATCTGG	4746
ATGAGG	5084
ATGACG	1402
ATGAGC	3635
<u>ATCAGC</u>	<u>3608</u>
ATCACC	3939
ATCAGG	3714

The two underlined ends, which are not regular and are relatively abundant in the rat genome, were chosen to design the following two DOP-PCR primers (bold letters are bases different from 6-MW primer):

ATCTGC: CCG ACT CGA GNN NNN NAT **CTG C**

ATCAGC: CCG ACT CGA GNN NNN NAT **CAG C**

To avoid contamination, we optimized the PCR conditions in order to obtain sufficient PCR products through only one round of PCR. We found that the concentration of magnesium (Mg^{2+}) in the PCR reaction was critical. The higher the Mg^{2+} , the more PCR products we could get, however, the smaller the size of the PCR products. The optimal Mg^{2+} was 2.5-3.5 mM for our purpose, which was two-fold the concentration that was used for ordinary PCR. Following the new protocol, we can obtain sufficient PCR products with an average size of 500 bp after one round of PCR, which were free of contamination. The new protocol also reduces the chance of having repeats at the ends of the inserts. Therefore the technical difficulties we had at the beginning of the project were resolved. Our protocol should also be helpful to other investigators in this field.

Subtopic *Mcs3*

The third potential locus that controls the resistance phenotype of mammary carcinogenesis in the Cop rat is termed *Mcs3*, which is localized to the upper-central region of chromosome 1. In BC1 we obtained a lod score of 1.7, which is close to a suggestive linkage (1.9). The "locus" locates near marker D1Wox6 (Figure 1). We then tested the chromosome 1 markers around *Mcs1* in F2 and BC2. The result showed a combined peak lod score of 5.1 in an interval of ≈ 30 cM.

Subtopic *Mcs4*

The loci *Mcs1* through *Mcs3* were associated with the resistance phenotype because animals with 0-1 tumors retained an allele from the resistance strain (Cop) at the loci and animals with many tumors retained both alleles from the susceptible strain (WF). In contrast, *Mcs4* on chromosome 8 was associated with the tumor enhancer phenotype because the locus showed no Cop allele in the low tumor number group but always a Cop allele in the high tumor number group, this locus was thus believed to contain a tumor enhancer gene from the Cop rat. This locus showed a lod score of 1.1 in BC1, which is below the suggestive threshold. However, we pursued the study of this locus since its effect was the opposite of the other three *Mcs* loci. There was also a small peak with a parametric lod score of 1.1 on chromosome 20 (data not shown). No other chromosomal regions yielded a lod ≥ 1 .

A combined analysis of data from BC1, F2 and BC2 showed *Mcs4* has a peak lod of 5.11 in an interval of approximately 15 cM.

Thus, there are 4 loci affecting the carcinoma phenotype in the Cop rat. The *Mcs* loci are summarized in Table 1. Since *Mcs1* has a much higher lod score than the other loci, we think it is the major gene controlling the mammary carcinoma susceptibility in the Cop rat, therefore our effort for positional cloning is focused on *Mcs1*.

Objective 4. Isolate coding sequences from P1 or cosmid clones containing *Mcs* phenotype-linked SSRs for determination of homologous regions in the human genome and possible correlation with already identified human genes.

We did not get a chance to start this experiment as our major efforts have been focused on isolating more SSR markers to fine-map the *Mcs* loci. However, this experiment may not be necessary for all the loci. As the rat genome project advances, we may be able to correlate our loci to some already identified genes, or we may be able to find human and mouse homologs of the *Mcs* loci by identifying homologous regions as defined by other genes mapped close to *Mcs*. We have obtained some homology information about *Mcs3* (see below). But we do not have enough evidence to define the homologous region of *Mcs1* and *Mcs2*.

The *Mcs3* region was determined to be homologous to mouse chromosome 7 and is likely to be homologous to one of several human chromosome regions such as 11p, 11q, 15q, or 19q. From our mapping data in BC1, the *Mcs3* locus lies between marker D1Mit11 (R260) and D1Mit2 (R1301), which is flanked by gene markers *Klk1* (Kallikrein 1, renal/pancreas/salivary, GenBank no: M19647, D1Wox18) and *Omp* (Olfactory marker protein, D1Mgh19) (<http://ratmap.gen.gu.se>). These two genes have already been mapped in the mouse and human. From the mouse genome data base at <http://www.informatics.jax.org> and human genome data base at <http://gdbwww.gdb.org>, we knew that *Klk1* maps to mouse chromosome 7 at 23.0 cM and human chromosome 19q13, and *Omp* maps to mouse chromosome 7 at 48.0

cM and human chromosome 11q14-21. The genes between KIK1 and Omp in the mouse genome, such as Oca2, Tyr, Hbb, were also known to be on rat chromosome 1 by somatic cell hybrid study (see <http://ratmap.gen.gu>), so that we deduced that the *Mcs3* was homologous to mouse chromosome 7 from 23.0 cM to 48.0 cM on the mouse genetic map. The Hbb gene (Hemoglobin, beta) was physically located on rat chromosome 1q22, implying that *Mcs3* is also near 1q22. However, these genes map to different chromosomes in the human genome, such as 11q21, 15q11. Thus by a comparison of the rat and mouse gene maps, we determined that the *Mcs3* region is homologous to mouse chromosome 7q and is conserved between the rat and mouse. A clearer human homologous region will be defined when more genes are mapped in the rat genome.

5. Test for loss of heterozygosity of *Mcs*-linked SSR markers in mammary tumors of two independent F1 hybrids following radiation and DMBA tumor induction.

Polymorphic markers throughout the rat genome were tested for loss of heterozygosity in radiation- and DMBA-induced tumors from (WF X Cop)F1 animals. No allelic imbalance was detected in the mapped locations of *Mcs1* through *Mcs4*. However, a scan of the genome revealed random allelic imbalance in the radiation-induced tumors. In addition, a non-random LOH on chromosome 1 in the DMBA-induced tumors was documented, but the position at which high LOH was observed was around the *H-ras* locus, which is 50 cM away from *Mcs3*. The result suggests that loss of *Mcs* alleles may not be required for the formation of

mammary tumors, implying that these genes are not functioning as typical tumor suppressor genes (such as *p53*).

CONCLUSIONS

We have found 4 loci (3 resistance and 1 enhancer) that showed linkage with the mammary carcinoma phenotype in the Cop rat. The locus on chromosome 2 (termed *Mcs1*) is believed to contain the major tumor resistance genes, while other loci such as *Mcs2* on chromosome 7, *Mcs3* on chromosome 1, and *Mcs4* on chromosome 8, represent relatively weaker genes. We will concentrate our efforts on further fine-mapping and positional cloning of *Mcs1* gene.

To summarize, the *Mcs* loci (or genes) we have identified through this project are listed in Table 1.

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Figure 1: QTL scan of rat chromosome 1, 2 and 7 in the (Cop x WF)F1 x WF backcross. The map and the scan were generated using the MAPMAKER and the MAPMAKER/QTL computer programs.

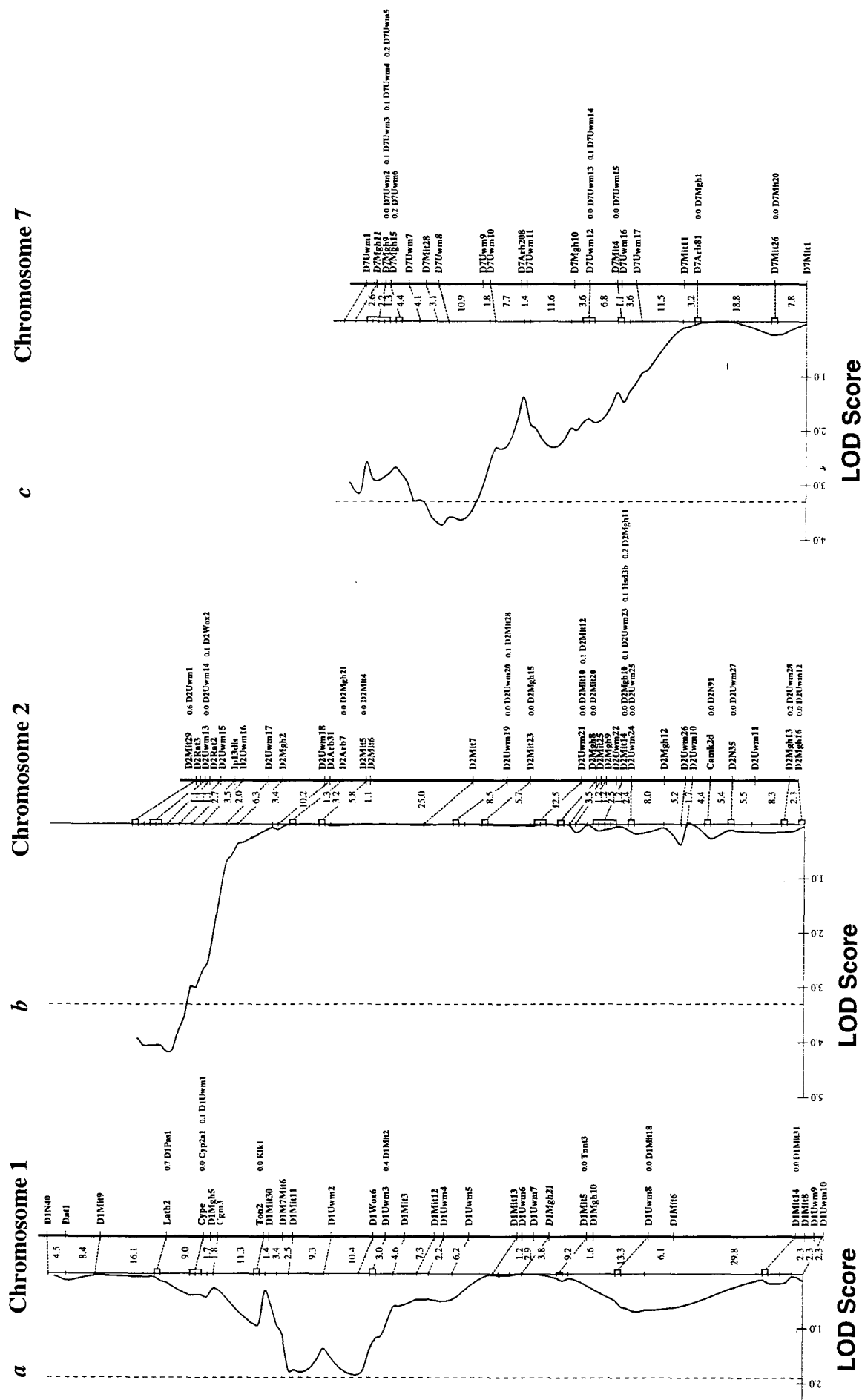


Table 1. Linkage of DNA markers to mammary carcinoma induction in three crosses

Table 1. Linkage of DNA markers to primary chromosome association in olive flies										
DNA marker	Δ Position, * cM	BC1		F2		BC2		Combined results		
		LOD _w	P value	LOD _w	P value	LOD _w	P value	-2 $\Sigma \ln P$	Combined P	LOD _w
Chromosome 1										
<i>Loxh2</i>	0.7	0.16	0.391	0.18	0.362	0.31	0.232	6.83	3.37E-01	0.65
<i>D1Pos1</i>	8.3	0.02	0.777							
<i>Cypc</i>	0.0	0.02	0.735							
<i>Cyp2a1</i>	0.0	0.27	0.261							
<i>D1Uwm1</i>	1.6	0.01	0.849							
<i>D1Mgh5</i>	1.8	0.49	0.132	1.00	0.032	0.19	0.344	13.07	4.20E-02	1.68
<i>Cypm3</i>	11.3	0.10	0.494							
<i>Tom2</i>	0.0	1.27	0.016	1.28	0.015	1.38	0.012	25.52	2.74E-04	1.93
<i>Kil1</i>	1.3	0.63	0.088							
<i>D1Mgh30</i>	3.4	0.17	0.375							
<i>D1M7Mgh69</i>	2.5	0.44	0.153							
<i>D1Mgh11</i>	9.3	1.66	5.67E-03	1.48	9.15E-03	1.55	7.52E-03	29.51	4.86E-05	4.69
<i>D1Uwm2</i>	10.4	0.59	0.099	0.90	0.042	1.84	3.59E-03	22.22	1.10E-03	3.33
<i>D1Wox6</i>	3.0	2.15	1.66E-03	0.84	0.049	2.15	1.66E-03	31.64	1.92E-05	5.14
<i>D1Uwm3</i>	0.4	1.27	0.016							
<i>D1Mgh2</i>	4.2	1.21	0.018	0.33	0.216	3.07	1.68E-04	28.48	7.62E-05	4.61
<i>D1Mgh3</i>	7.3	0.56	0.109	0.54	0.114	1.82	3.81E-03	19.92	2.87E-03	2.92
<i>D1Mgh12</i>	2.2	0.51	0.125							
<i>D1Uwm4</i>	6.2	0.54	0.114							
<i>D1Uwm5</i>	12.0	0.49	0.134							
<i>D1Mgh13</i>		0.01	0.828							
Chromosome 2										
<i>D2Mgh29</i>	0.6	3.61	4.59E-05	1.73	4.74E-03	9.22	7.31E-11	77.36	1.25E-14	14.56
<i>D2Uwm1</i>	0.5	3.80	2.88E-05							
<i>D2Rat3</i>	1.1	4.40	6.77E-06	2.62	5.07E-04	8.28	6.70E-10	81.23	1.99E-15	15.30
<i>D2Uwm13</i>	0.0	3.83	2.68E-05	2.35	9.96E-04	8.94	1.41E-10	80.24	3.18E-15	15.12
<i>D2Uwm14</i>	0.0	4.31	8.31E-06	1.83	3.65E-03	9.09	9.85E-11	80.70	2.56E-15	15.23
<i>D2Wox2</i>	1.1	4.32	8.09E-06	2.53	6.45E-04	7.53	3.87E-09	76.88	1.57E-14	14.38
<i>D2Rat2</i>	2.7	3.91	2.22E-05	2.87	2.76E-04	7.90	1.61E-09	78.32	7.96E-15	14.68
<i>D2Uwm15</i>	3.5	3.99	1.83E-05	2.11	1.85E-03	4.85	2.31E-06	60.36	3.81E-11	10.95
<i>ip13a3a</i>	2.0	2.56	5.94E-04	1.14	0.022	4.49	5.41E-06	46.75	2.10E-08	8.19
<i>D2Uwm16</i>	6.3	2.53	6.34E-04	1.23	0.018	3.64	4.20E-05	42.92	1.21E-07	7.40
<i>D2Uwm17</i>	3.4	0.77	0.059	0.95	0.037	3.72	3.51E-05	32.77	1.16E-05	5.44
<i>D2Mgh2</i>	10.2	0.21	0.331							
<i>D2Uwm18</i>		0.01	0.793							
Chromosome 7										
<i>D7Uwm1</i>	2.5	2.40	8.79E-04							
<i>D7Mgh11</i>	2.2	2.58	5.69E-04	0.11	0.480	0.96	0.035	23.12	7.59E-04	3.65
<i>D7Mgh9</i>	0.0	2.27	1.21E-03	0.24	0.290	0.55	0.113	20.27	2.48E-03	3.06
<i>D7Mgh15</i>	4.4	2.84	3.02E-04	0.21	0.330	0.21	0.32	20.71	2.07E-03	3.26
<i>D7Uwm7</i>	4.1	3.07	1.68E-04	0.13	0.435	1.06	0.028	26.20	2.04E-04	4.26
<i>D7Mgh28</i>	3.1	3.38	7.91E-05	0.16	0.386	3.02	1.92E-04	37.91	1.17E-06	6.56
<i>D7Uwm5</i>	10.9	2.77	3.51E-04							
<i>D7Uwm9</i>	1.8	1.42	0.011							
<i>D7Uwm10</i>	7.7	1.57	7.10E-03	0.61	0.094	3.94	2.06E-05	36.20	2.52E-06	6.12
<i>D7Arb208</i>	1.4	1.13	0.023	0.23	0.304					
<i>D7Uwm11</i>	11.6	1.86	3.38E-03	0.56	0.109	5.52	4.64E-07	44.98	4.72E-08	7.94
<i>D7Mgh10</i>	3.6	1.85	3.53E-03	0.45	0.149	0.95	0.036	21.75	1.34E-03	3.25
<i>D7Uwm12</i>	0.0	1.10	0.024	0.46	0.144					
<i>D7Uwm13</i>	0.0	1.32	0.014							
<i>D7Uwm14</i>	0.7	1.24	0.017							
<i>D7Mgh4</i>	0.0	1.28	0.015							
<i>D7Uwm15</i>	1.1	1.34	0.013							
<i>D7Uwm16</i>	3.6	0.76	0.061							
<i>D7Uwm17</i>	11.5	0.75	0.064							
<i>D7Mgh11</i>		0.01	0.866							
Chromosome 8										
<i>D8Mgh14</i>	11.3	0.33	0.220							
<i>D8Mgh1</i>	4.7	0.62	0.092							
<i>D8Mgh4</i>	9.5	0.57	0.105							
<i>D8Mgh7</i>	1.6	0.52	0.121	0.06	0.596	1.81	3.84E-03	16.38	1.18E-02	2.39
<i>D8Mgh6</i>	1.9	0.98	0.033	0.16	0.392	1.34	0.013	17.38	7.98E-03	2.48
<i>D8Mgh16</i>	0.0	0.69	0.075	0.22	0.318	2.18	1.54E-03	20.42	2.33E-03	3.09
<i>D8Mgh13</i>	10.4	1.02	0.030	0.08	0.537	1.80	4.01E-03	19.29	3.69E-03	2.90
<i>D8Mgh3</i>	1.7	0.44	0.155	0.63	0.088	4.04	1.60E-05	30.68	2.92E-05	5.11
<i>D8Mgh4</i>	8.5	0.69	0.074	0.58	0.103	3.56	5.15E-05	29.50	4.89E-05	4.83
<i>D8Mgh5</i>		0.65	0.083	0.40	0.175	3.94	2.04E-05	30.06	3.82E-05	4.99

* Δ Position is given as the cM distance (using the Kosambi function) between that marker and the marker below it in the table. Distances were determined in the genome scan using BC1 and vary slightly in the other two crosses. For each chromosome, only the subset of markers in the regions of the QTLs are shown.

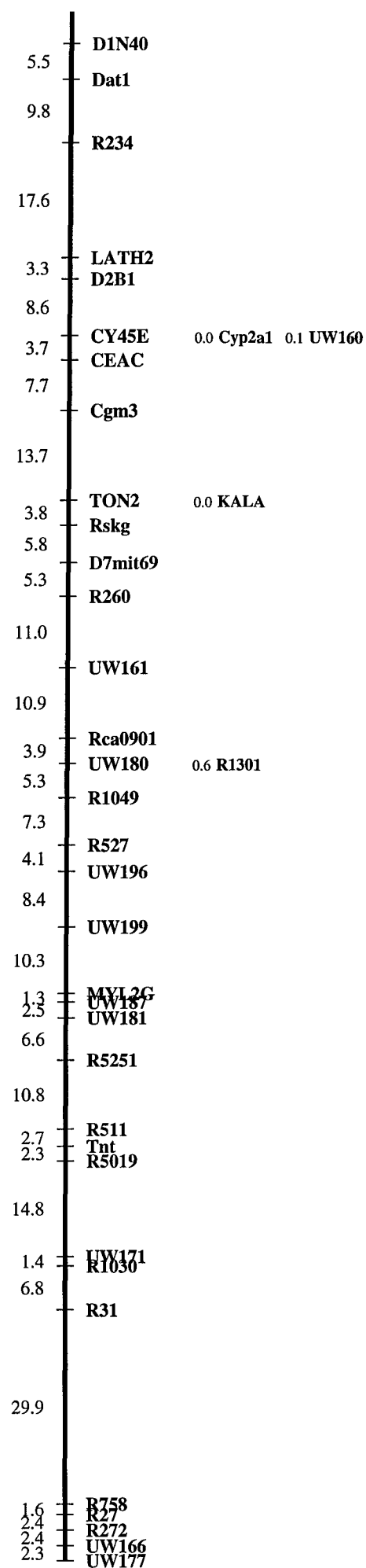
** Combined results were calculated as described (Fisher, 1973). LOD_w was estimated from Qlink Z scores as described (Kruglyak and Lander, 1995).

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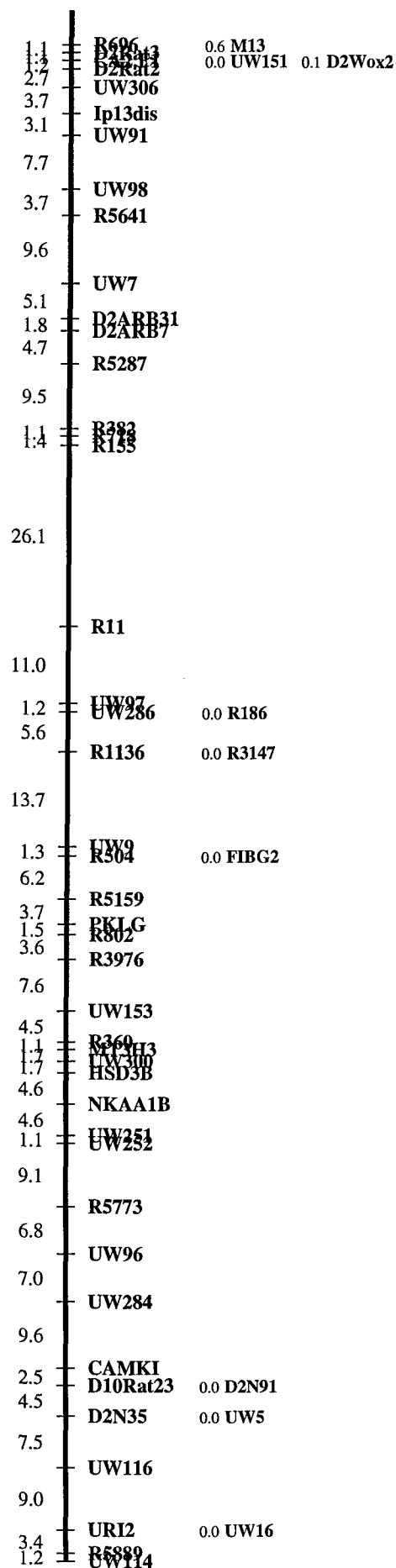
APPENDIX

Genetic linkage map of rat genome constructed in the (WF X Cop)F₁ X WF
backcross.

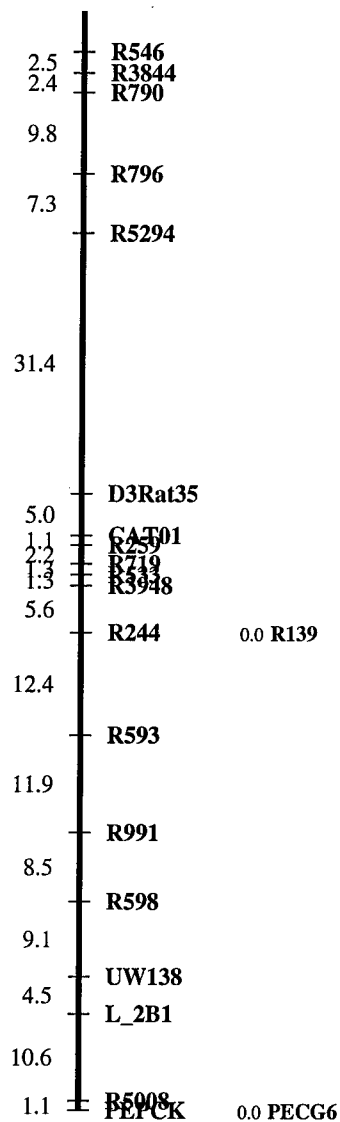
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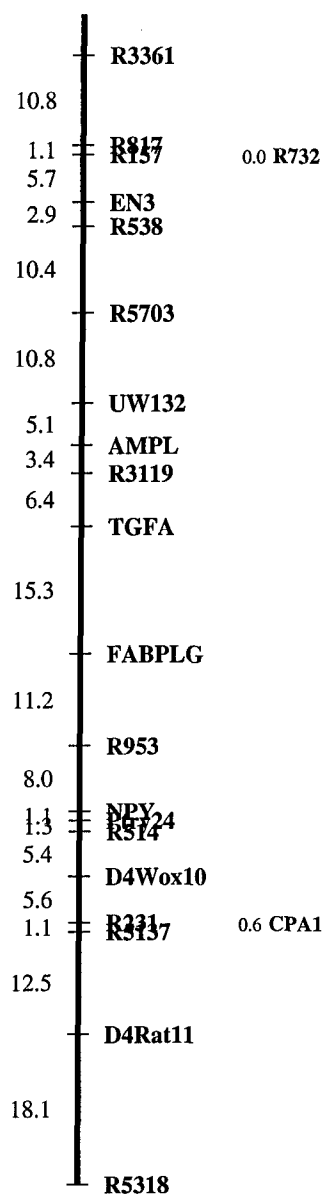
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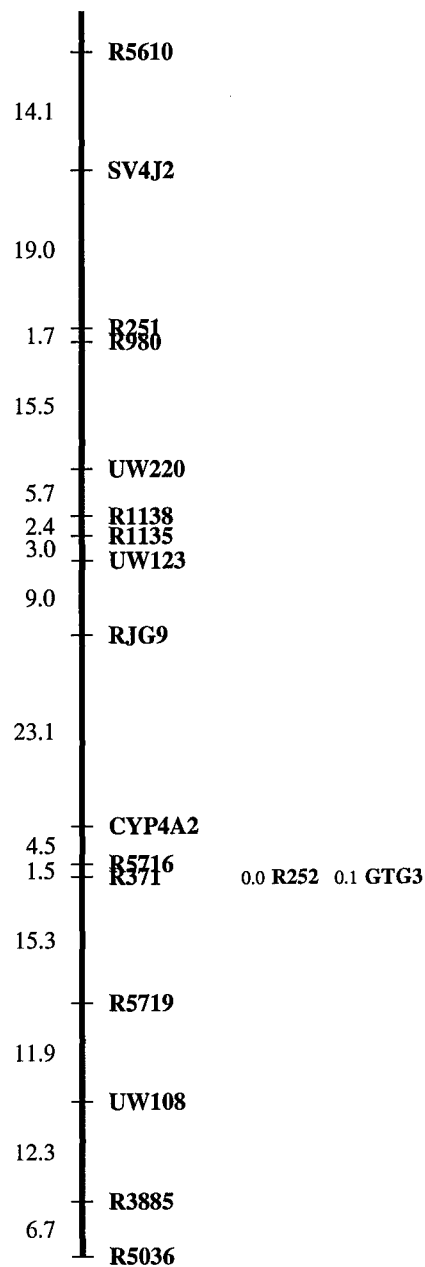
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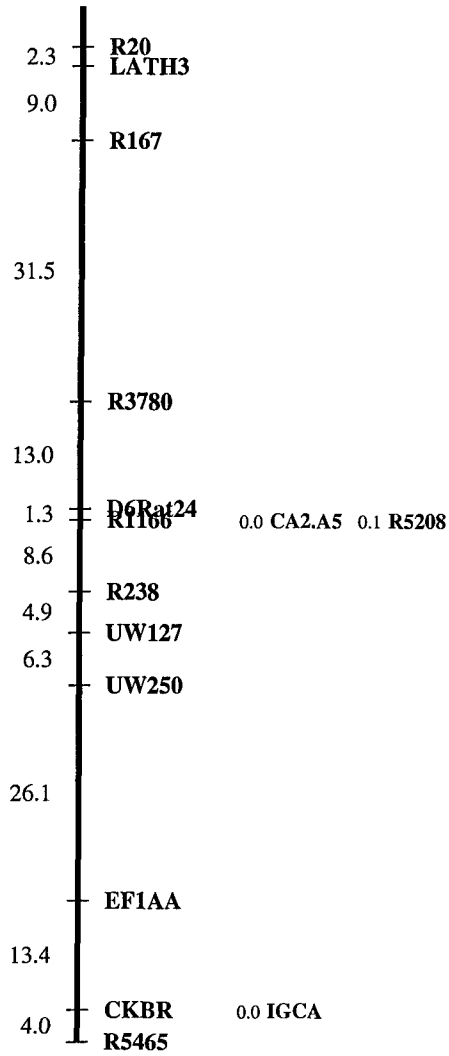
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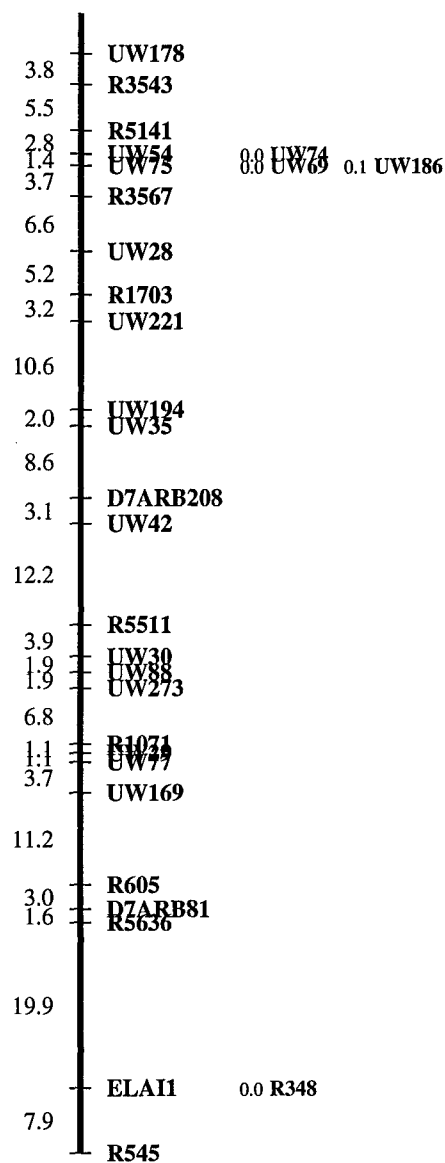
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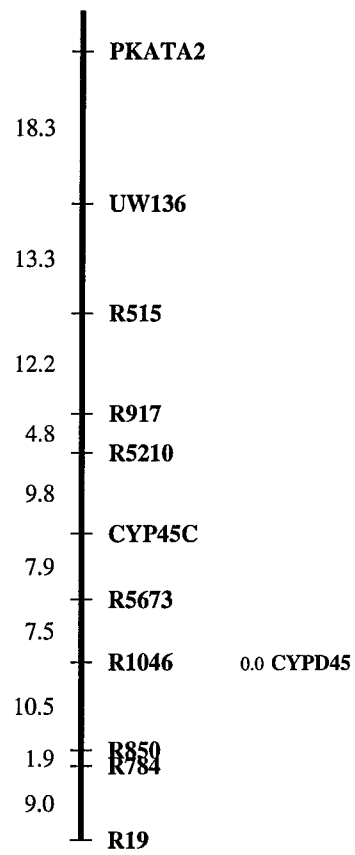
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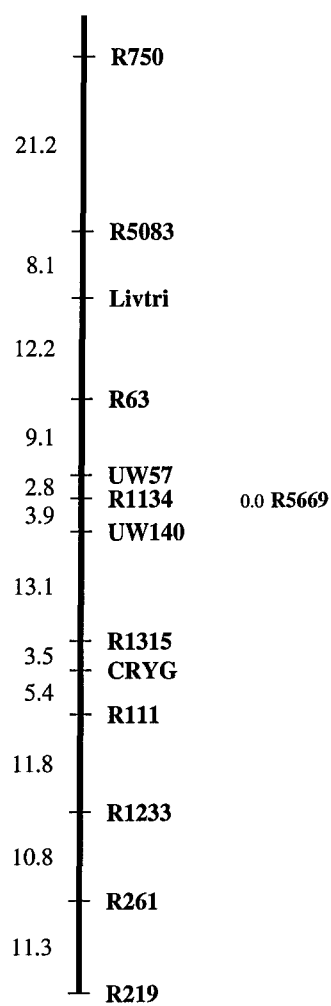
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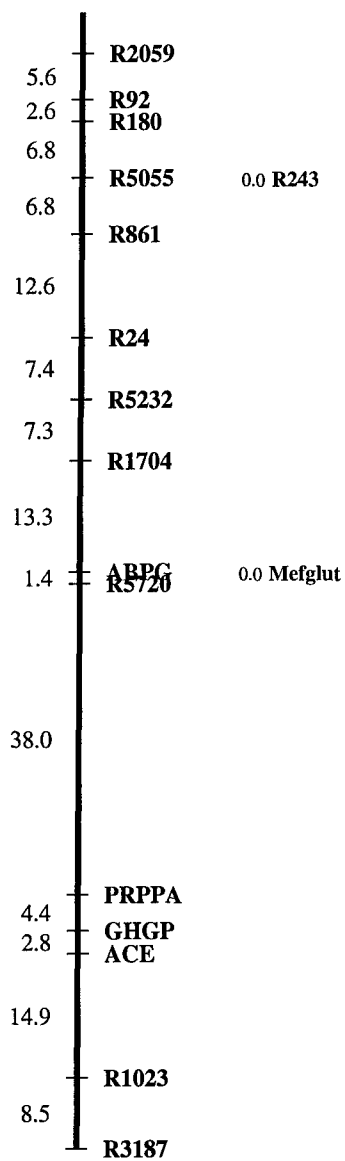
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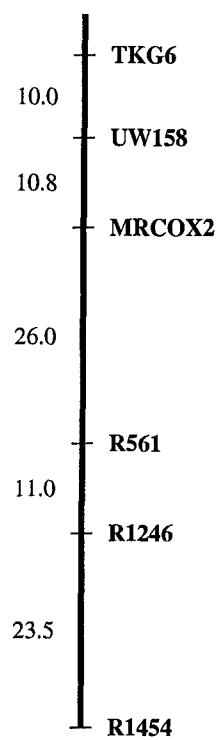
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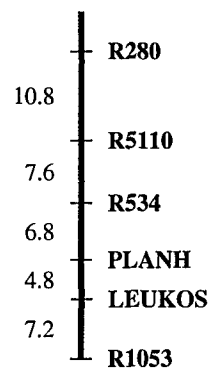
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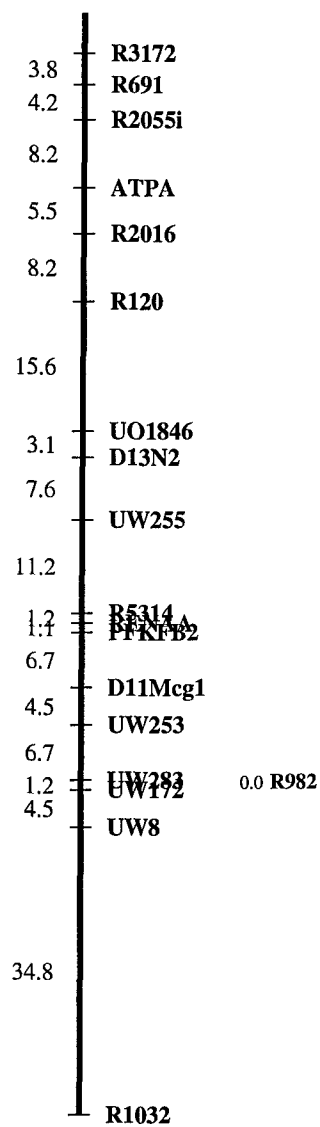
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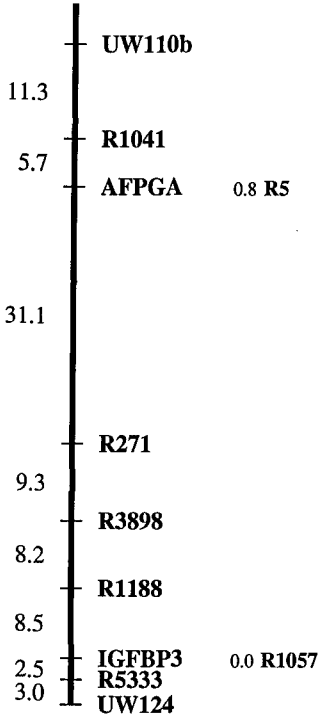
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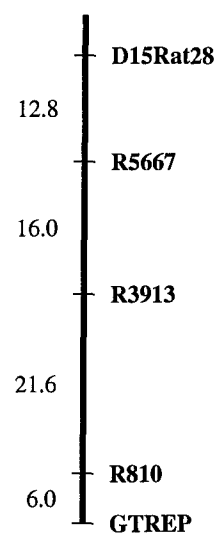
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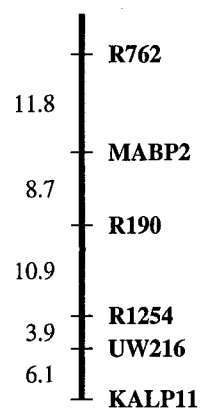
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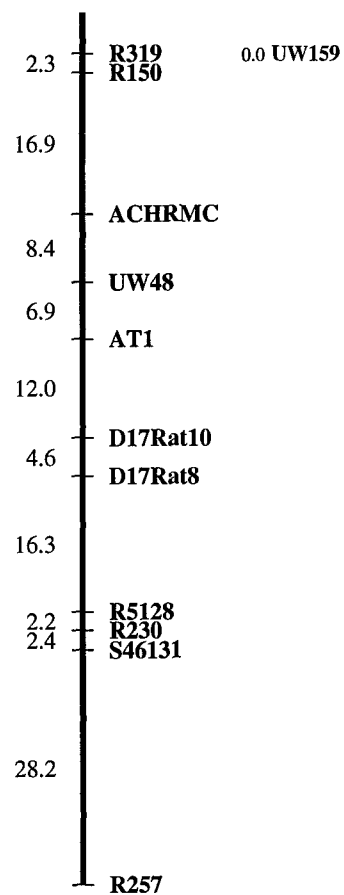
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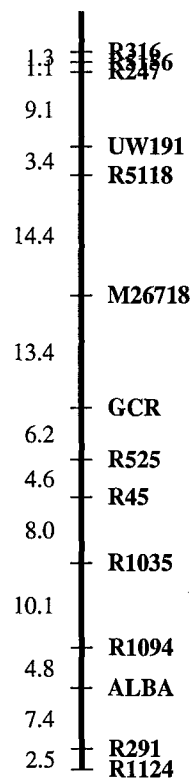
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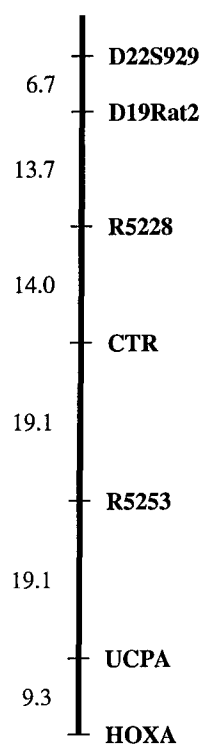
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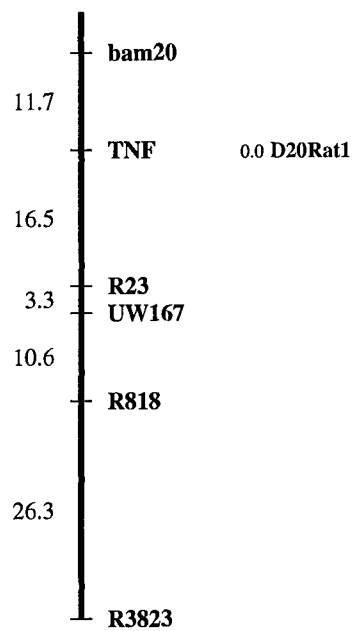
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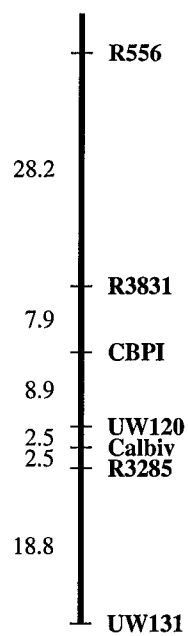
Chromosome c19



Chromosome c20



Chromosome cx



Publication

Shepel, L.A., Lan, H., Haag, J.D., Brasic, G.M., Gheen, M.E., Simon, J.S., Newton, M.A. and Gould, M.N. Genetic identification of multiple loci that control breast cancer susceptibility in the rat. In preparation, 1997.

Abstracts

Gould, M.N., Benton, M., Lan, H., Haag, J.D. and Shepel, L. Genetics of mammary cancer susceptibility in the rat: Potential relevance to the prevention and genetics of human breast cancer. AACR Special Conference in Cancer Research: Basic and Clinical Aspects of Breast Cancer. March 7-12, 1997, Keystone Resort, Keystone, CO.

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